Cloning, functional expression and mRNA distribution of an inwardly rectifying potassium channel protein

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Abstract In GH_3/B_6 cells at least two different inward K^+ currents are observed that are regulated by thyrotropin-releasing hormone and somatostatin, respectively. Using a polymerase chain reaction based approach a cDNA was isolated and functionally expressed in human embryonic kidney cells that encodes an inward rectifier K^+ channel, rIRK3, with a predicted molecular mass of 49.7 kDa. Corresponding transcripts of 2.6 kb have been detected in rat brain, pituitary and GH_3/B_6 cells. In situ hybridization revealed that rIRK3 mRNA is distributed throughout the brain and occurs predominantly in the piriform cortex, indusium griseum, supraoptic nucleus, facial nucleus and cerebellar Purkinje cells.

Key words: GH_3/B_6 cells; Hybridization, in situ; Oocyte expression; Human embryonic kidney cell expression; Anterior pituitary; Prolactin secretion

1. Introduction

Neuropeptide-controlled prolactin secretion in GH_3/B_6 anterior pituitary cells is accompanied by changes in the cytosolic CA^{2+} concentration and plasma membrane electrical properties [1]. Thyrotropin-releasing hormone (TRH) causes a biphasic release of hormone that correlates with a transient rise in cytosolic CA^{2+} levels by inositol 1,4,5-trisphosphate-mediated release from internal stores and by a sustained CA^{2+} influx into the cytoplasm from the extracellular space [2]. Somatostatin (SST) mediates an inhibition of the secretory process with a concomitant decrease in internal CA^{2+} levels due to impaired influx [3]. Thus, voltage-gated CA^{2+} channels are critically involved in the regulation of cytosolic CA^{2+} levels and hence play a major role in secretion. The activity of these channels is, in turn, influenced by changes in the membrane potential, i.e. depolarization increases channel open probability and promotes CA^{2+} influx, while hyperpolarization leads to channel closure and thereby reduces CA^{2+} influx [4]. Two different K^+ currents have been described in GH_3/B_6 cells that are modulated by TRH and SST, respectively. The activity of a voltage-dependent inward K^+ current contributes to the membrane resting potential and is inhibited by TRH [5]. The decreased K^+ conductance causes membrane depolarization and is thought to facilitate CA^{2+} influx through stimulation of voltage-gated CA^{2+} channels. In contrast, SST induces membrane hyperpolarization by increasing K^+ conductance through G protein-mediated opening of 55 pS K^+ channels, thereby reducing CA^{2+} influx [6]. Recent cDNA cloning experiments have revealed that both inward rectifier and G protein-operated K^+ channels are members of a family of K^+ channel proteins that also includes ATP-sensitive K^+ channels [7–15]. This family differs from the family of voltage-gated K^+ channels of the delayed rectifier type by the presence of only two instead of six transmembrane regions. In order to identify the K^+ channel proteins that are involved in the neuropeptide-induced modulation of prolactin secretion, a polymerase chain reaction approach was used to isolate and characterize a cDNA from a GH_3/B_6 cell library which encodes an inwardly rectifying K^+ channel.

2. Materials and methods

2.1. Isolation and sequencing of cDNA

Degenerate deoxynucleotide primers were synthesized based on homologous regions of the nucleotide sequences of mouse IRK1, rat ROMK1 and rat GIRK1 channels [7,10,11] *P1(forw.)*: CCGAATTCGAA(G)ACIC(G)AA(G)GC(T)IACIATT(C)(A)GGITAT(C)GG; *P2(forw.)*: CCGAATTCGCIGTIATT(C)(A)G(T)CIAT(A)GAGGGAC(T)GGIAA; *P3(rev.)*: CCGAATTCTGA(G)TCC(T)AG(A)G(T)(A)GGG(A)(T)ATGG(A)T(A)A(A)T(C)TCICCT(C)TC. Polymerase chain reactions (PCR) using cDNA templates from rat GH_3/B_6 cells generated two fragments of 150 bp (P2 and P3) and 330 bp (P1 and P3). Sequence analysis (using the GCG software package; GCG, Madison, WI, USA) showed that the fragments were identical in the region of overlap and revealed an 80% similarity with the corresponding nucleotide sequence of the murine IRK1 channel. The [^{32}P-dCTP]-labelled 330 bp-fragment was used to screen a rat GH_3/B_6 cell cDNA library constructed in phage lambda ZAP (1×10^6 plaque forming units; Stratagene, Heidelberg, Germany). Three positive clones were identified and the plaque purified. In vivo excision and rescue of pBluescript SK(–) from the positive lambda ZAP clones were performed according to the manufacturer's instructions. We selected one clone containing an 1.8 kb insert for further characterization. Sequencing of the DNA of both strands with an automated Applied Biosystems Model 373 A DNA Sequencer and dye terminator showed that the clone lacked part of the coding region at the 5' end. In order to obtain a full-length cDNA, an anchored PCR with either a T3 primer, CCGAATTCGCAATTAACCCTCACTAAAGGG, or a T7 primer, CCGAATTCAATACGACTCACTATAGGGCGA, and a reverse primer, CCGAATTCACATGCATGATACACGGTTTGG, specific for a region close to the 5' end of the 1.8 kb cDNA was carried out using 1×10^9 phages from the GH_3/B_6 cDNA library. A 0.9 kb fragment was isolated that contained the translation start codon. The 1.8 and the 0.9 kb fragments were blunt-end ligated by an internal *Sma*I restriction site and cloned into a pBluescript SK(+) vector. An *Eco*RI fragment including the entire cDNA was cloned into the expression vector pGEMHE containing 5' and 3' UTRs of the β globin gene from *Xenopus laevis* [16].

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The nucleotide sequence(s) presented here has (have) been submitted to the EMBL/GenBank database under the accession number no. X87635.

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2.2. Northern blot analysis

Total RNAs from various tissues were extracted using the RNAzol B-Method (Biotecx Lab. Inc., Houston, TX, USA). Equal amounts (50 μg) of total cellular RNA were separated on a 1.2% agarose-formaldehyde gel, transferred to a Hybond-N nylon membrane (Amersham, Braunschweig, Germany) and hybridized with the cloned ^{32}P-labelled cDNA. A β-actin cDNA probe was used to demonstrate that all lanes contained comparable amounts of RNA. After a final wash in 0.1 × SSC, 0.1% SDS, at 65°C for 60 min, the blots were analyzed in a BioImager (Fujix BAS 3000, Fuji, Japan).

2.3. Functional expression in Xenopus laevis *oocytes and human embryonic kidney cells*

After linearization of the cDNA clone at the NheI site in the polylinker region, cRNA was synthesized in vitro using a T7 RNA-polymerase kit (Promega Biotec, Madison, WI, USA) and pressure injected into stage V *Xenopus laevis* oocytes (50 nl, 10 ng/μl). Voltage-clamp measurements were done with a conventional two electrode voltage-clamp (CA100, VF1800, VF180, Biologic, Claix, France) at room temperature. Stimulation and data analysis were performed with a CED1401-patch clamp software (Cambridge Electronic Design, Cambridge, UK).

Human embryonic kidney HEK/A293 cells were plated on poly-L/D-lysine coated cellocates (Eppendorf, Germany) and kept in Dulbecco's modified Eagle medium/Ham's F12 medium supplemented with penicillin/streptomycin (50 U/ml, 50 μg/ml), L-glutamine (4 mM) and fetal calf serum (10%) at 37°C in 5% CO_2. Electrophysiological measurements were made from cells 1 day after plating and 4–6 h after injection of cRNA (20 ng/μl) with an automated microinjection system (Zeiss, Germany). Patch pipettes filled with internal solution (in mM: 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 2.5 EGTA, 10 HEPES, pH 7.3) had a resistance of 3–4 MΩ. External solution in mM: 140 KCl, 4 $MgCl_2$, 1 $CaCl_2$, 2.5 EGTA, 30 glucose, 10 HEPES, pH 7.3, also containing 0.5 μM tetrodotoxin. Stimulation, data collection and analysis were carried out with an EPC9 patch clamp amplifier and Pulse/Pulsefit software (Heka, Lambrecht, Germany). Experiments were performed at room temperature.

2.4. In situ hybridization

12 mm cryostat cross-sections from rat brains were fixed with 4%

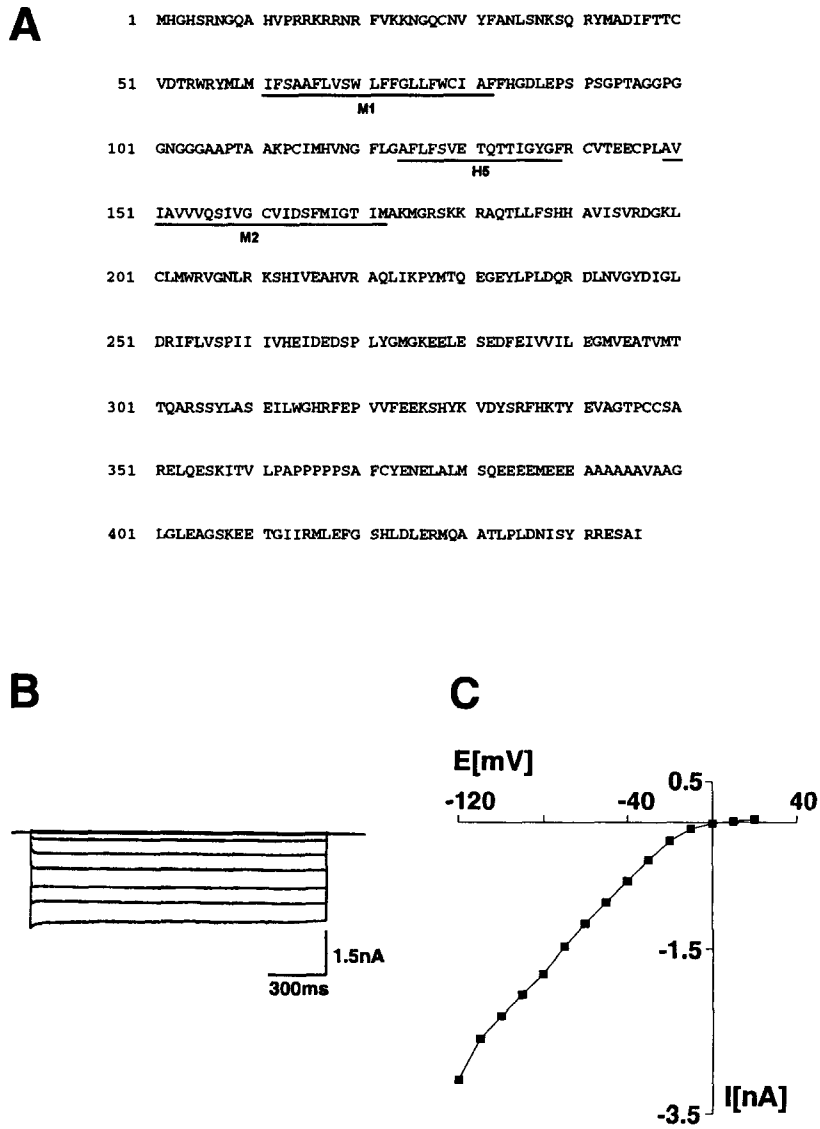

Fig. 1. (A) Amino acid sequence of rIRK3 cDNA. Amino acid residues are numbered from the initiating methionine. Putative transmembrane segments (M1 and M2) and pore forming region (H5) are marked by lines below the sequence. (B) Inwardly rectifying current in a HEK/A293 cell expressing rIRK3. Current traces elicited by 1.5 s voltage steps to 20, 0, −20, −40, −60, −80, −100, −120 mV from a holding potential of 0 mV. (C) Current–voltage relationship obtained from the same HEK cell as shown in (B). Currents were elicited by voltage steps to test potentials ranging from 20 mV to −120 mV in −10 mV increments. Peak current amplitudes are plotted against the membrane potential.

paraformaldehyde and hybridized with fluorescein-labelled cRNA in antisense or, for control, in sense orientation. Following the hybridization the slides were washed and treated with RNase A and cRNA was detected by synthesis of chromophores through alkaline phosphatase activity coupled to an anti-fluorescine antiserum using the standard protocol (Amersham, Braunschweig, Germany). For the control, adjacent sections were hybridized with sense riboprobes or digested with RNase A (100 mg/ml), 30 min, 37°C. No hybridization signals were observed with these controls.

3. Results and discussion

Reverse transcriptase polymerase chain reaction amplification of GH_3/B_6 cell RNA using primers P1 and P3 or P2 and P3 led to the identification of two overlapping DNA fragments with identical DNA sequence. Using the fragments as probes, a cDNA clone of 2561 bp was isolated. Its nucleotide sequence displays a stretch of adenine residues at the 3'-end that is preceded by a canonical AATAAA signal, indicating that the entire mRNA 3'-end has been cloned (data not shown). Since the length of the cDNA corresponds well to the size of its mRNA (Fig. 2), most if not all of the mRNA 5'-end has also been cloned. The cDNA specifies a single open reading frame that is preceded by an in frame translational stop codon and encodes a protein of 446 amino acids with a predicted molecular mass of 49.7 kDa. Comparisons of the deduced amino acid sequences with other K^+ channel proteins (result not shown) indicates that it belongs, as expected, to the family of K^+ channels with two putative membrane spanning domains which includes inward rectifier, G protein-operated and ATP-regulated K^+ channels [7–15]. The deduced amino acid sequence (Fig. 1A) shows highest similarity to the mouse IRK3 inward rectifier channel [12] indicating that it represents the rat homologue, and hence, has tentatively been termed rIRK3. The sequence of rIRK3 is almost identical to an rat inward rectifier, BIR11 [15] except for 3 amino acids and for the last fourteen amino acids at the carboxy-terminus that are missing in BIR11.

Table 1
Localization of rIRK3 mRNA in the rat brain

Brain region	Rel. expr.	Brain region	Rel. expr.
Telencephalon		Diencephalon	
Olfactory system accessory olfactory nucl.	+	Thalamus	
Olfactory tubercle	++	Reticular nucl.	+
Anterior olfactory nucl.	+	Geniculate nucl.	+
Allocortex		Medial habenular nucl.	+
Piriform cortex	+++	Lateral habenular nucl.	+
Cingulate cortex	++	Paraventricular nucl.	+
Insular cortex	+	Laterodorsal thalamic nucl.	+
Orbital cortex	+/–	Paratenial thalamic nucl.	+
Isocortex		Centromedial thalamic nucl.	+
Frontal cortex	+/–	Mediodorsal thalamic nucl.	+
Parietal cortex	+/–	Reunien thalamic nucl.	+
Occipital cortex	++	Anterior thalamic nucl.	+
Temporal cortex	++	Hypothalamus	
Hippocampus		Median proptic nucl.	+
CA1 of cornu ammonis	++	Median preoptic area	++
CA3 of cornu ammonis	++	Lateral preoptic area	+
Dentate gyrus	+++	Anterior hypothalamic area	+
Hilus of dentate gyrus	++	Supraoptic nucl.	+++
Subiculum	++	Suprachiasmatic nucl.	+
Entorhinal cortex	++	Paraventricular nucl.	+
Amygdala		Arcuate nucl.	+
Nucl. lateral olfact. tract	+	Lateral hypothalamic area	+
Medial nucl.	+	Pretectum and midbrain	
Cortical nucl.	+	Ventral tegmental area	++
Central nucl.	+	Substantia nigra, pars retic.	++
Basolateral amygd. nucl.	+	Lower brainstem	
Claustrum	+	Superior colliculus	+/–
Endopiriform nucl.	++	Inferior colliculus	+
Bed. nucl. stria terminalis	+	Interpeduncular nucl.	++
Indusium griseum	+++	Raphe nucl.	++
Tenia tecta	+++	Preolivary nucl.	++
Septum		Lateral superior olive	+
Lateral septal nucl.	+	Pontine nucl.	++
Septohippocampal nucl.	+	Occulomotor nucl.	++
Nucl. diagonal band		Central grey	+
Vertical part	+	Mesencephalic nucl.	+
Horizontal part	+	Vestibular nucl.	++
Magnocellular preoptic nucl.	+	Cochlear nucl.	+
Striatum		Trigeminal nucl.	++
Globus pallidus	+/–	Facial nucl.	+++
Ventral pallidum	+/–	Cerebellum	
Caudate putamen	+/–	Purkinje cells	++
		Deep nucl.	++

The relative distribution of rIRK3 mRNA in the rat brain is described as: +/–, only few; +, most cells are weakely labelled in an area; ++, moderate and +++, strong labelling of cells in an area.

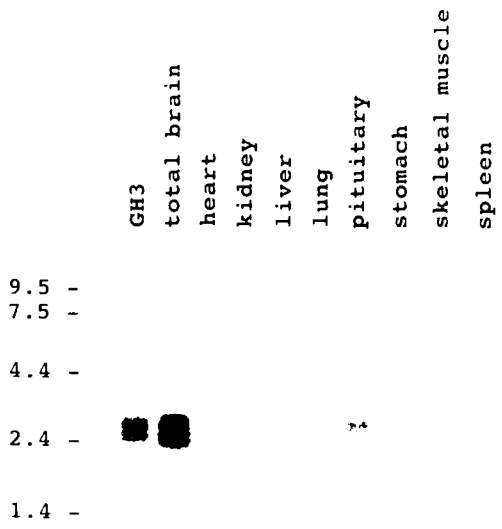

Fig. 2. Distribution of rIRK3 mRNA in various tissues by Northern blot analysis. The positions of the RNA size markers (in kb) are shown on the left side. The size of rIRK3 mRNA was determined to be 2.6 kb. The integrity of each RNA was confirmed by reprobing the same filter with a β-actin cDNA probe (data not shown).

Hyperpolarization of *Xenopus laevis* oocytes or human embryonic kidney cells that were previously injected with rIRK3 cRNA evoked large inward currents in isotonic K^+ solutions, while only marginal outward currents were observed. In both oocytes (data not shown) and HEK cells (Fig. 1B,C), the rIRK3 currents are partially activated at membrane potentials between –10 and –20 mV and inactivate to a small degree at very negative potentials. rIRK3 current amplitude and reversal potential depend on the external K^+ concentration (data not shown). Similar to other K^+ selective ion channels rIRK3 is blocked by Na^+, Ba^{2+} and Cs^+ ions in a voltage-dependent and reversible manner with EC_{50} values of 97 mM, 93 μM and 47 μM at –130 mV, respectively (data not shown). rIRK3-mediated currents are also partially blocked to 75% by high concentrations of tetraethylammonium ions (10 mM) but are not blocked by Ni^{2+} and Co^{2+} (data not shown). The deduced amino acid sequence of rIRK3 and the results from functional expression clearly indicate that rIRK3 does not mediate the SST-induced K^+ inward current observed in GH_3/B_6 cells, since this current can not be activated in the absence of the ligand [6]. Furthermore, co-injection of cRNA encoding somatostatin-receptor subtype 1, 2 and 3 [17–19] with rIRK3 cRNA into oocytes did not result in a SST-mediated modulation of rIRK3-induced currents (data not shown). It is also questionable whether rIRK3 corre-

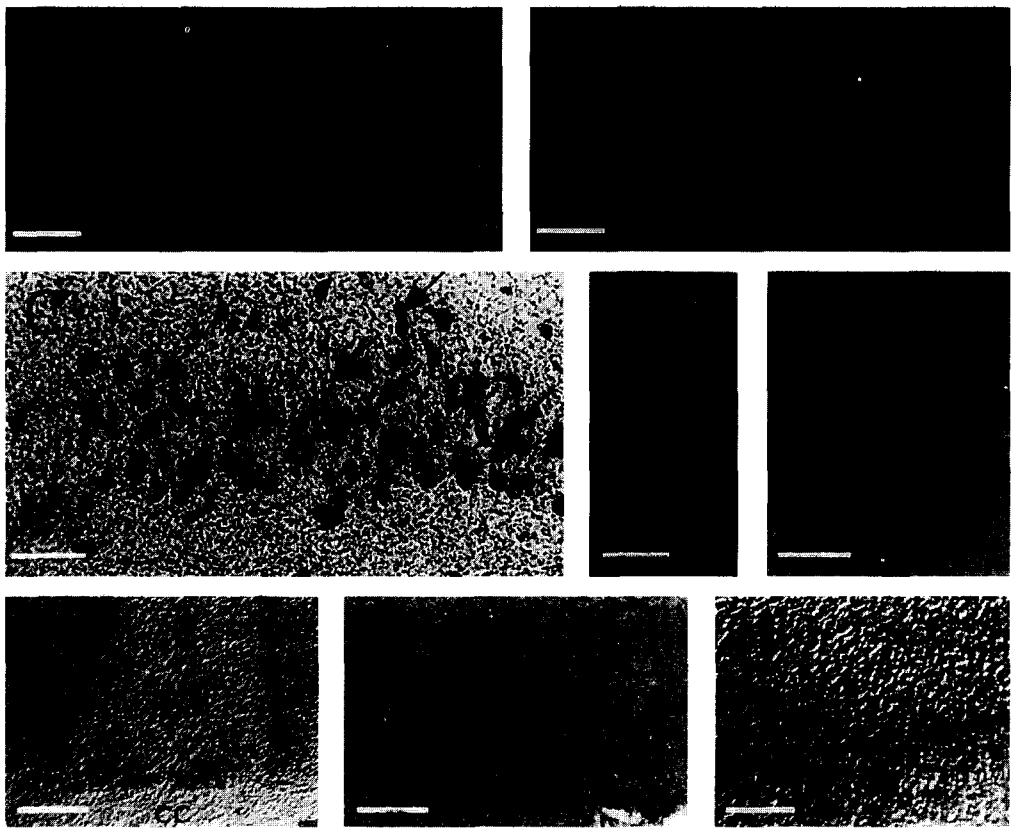

Fig. 3. In situ hybridization of fluorescein-labeled rIRK3 mRNA to cross sections of rat brain. (A and B) Bright field photomicrograph of cross sections through the hippocampal region hybridized to antisense or for a control to sense riboprobes, respectively. CA1, CA3, cornu ammonis, fields 1 and 3; DG, dentate gyrus, PoDG, polymorphic layer of dentate gyrus. (C) Phase contrast micrograph of part of the polymorphic layer of dentate gyrus. Arrows point to the dendrites of neurons that contain rIRK3 mRNA. (D and E) Localization of rIRK3 mRNA in the cerebellar and piriform cortex shown in bright field micrographs. The laminar structure of the hind limb area is indicated according to ref. 23. (F,G,H) Differential interference contrast micrographs showing the presence of rIRK3 transcripts in the indusium griseum, the facial nucleus and the Purkinje cell layer of the cerebellar cortex, respectively. IG, indusium griseum; cc, corpus callosum; GCL, granule cell layer; PCL, Purkinje cell layer; ML, molecular layer. Scale bars = 0.47 mm (A,B), 30 mm (C), 0.45 mm (D), 0.3 mm (E), 0.17 mm (F), 0.1 mm (G) and 70 mm (H).

sponds to the hyperpolarization-activated inward rectifier identified in GH_3/B_6 cells [5]. This channel displays a more pronounced inactivation than that produced by rIRK3 in oocytes and human embryonic kidney cells. In addition, co-injection of TRH-receptor cRNA [20] with that of rIRK3 into oocytes did not result in a TRH-mediated closure of rIRK3 channels. These results indicate either that recombinant rIRK3 channels differ from native ones, or that co-injection of additional components which are absent in oocytes or human embryonic kidney cells (such as an appropriate G protein) may be required. Alternatively, rIRK3 may represent an inward rectifier channel that has still not been identified, physiologically, in GH_3/B_6-cells. Another possibility would be the existence of an auxiliary β subunit of the inward rectifier channel mediating an inactivation similar to that of the native inward rectifier current in GH_3/B_6-cells. In the case of other known voltage-gated ion channels including the delayed rectifier family, inactivation due to a coexpressed β subunit has been shown [21].

RNA blot analysis demonstrates the presence of a 2.6 kb transcript in rat brain, pituitary and GH_3/B_6 cells. A larger transcript of about 4.4 kb has also been observed in skeletal muscle with low stringency hybridizations. However, with stringent washing conditions (0.1 × SSC, 65°C) the 4.4 kb band is not detected indicating that it likely represents a cross-hybridizing inward rectifier K^+ channel mRNA, probably that of rIRK2 [14].

In order to investigate the detailed distribution of rIRK3 mRNA in rat brain, in situ hybridization has been carried out. The rIRK3 gene is widely expressed in the rat central nervous system (Table 1, Fig. 3). It is found in the neocortex, the basal ganglia, several hypothalamic and thalamic nuclei, as well as in nuclei of the midbrain and brainstem, in cerebellar nuclei and in the Purkinje cells of the cerebellar cortex. The highest expression levels have been observed in the tenia tecta, indusium griseum, piriform cortex, facial and supraoptic nuclei and in the CA field and the dentate gyrus of the hippocampus. Higher magnification shows that almost all pyramidal cells of the CA fields and almost all of the granule cells of the dentate gyrus are intensely labeled (Fig. 3A). Also many neurons in the polymorphic layer of the dentate gyrus and a few cells in the molecular layer of the cornu ammonis contain significant levels of rIRK3 mRNA. Interestingly, in the CA3 field, rIRK3 mRNA is not only seen in the somata but also in the proximal dentrites of the pyramidal neurons (Fig. 3B). The dentritic location seems to be specific for the CA3 neurons, since it has not been observed in other brain regions. In the neocortex, layers II-VI are intensely stained by the probe (Fig. 3D,E). In particular, predominantly large cells that are likely to be pyramidal neurons are labeled by probe antisense RNA. Expression of the rIRK3 gene in the cerebellar cortex is almost exclusively confined to the Purkinje cells (Fig. 3F). Only very few other cells, scattered in the granule cell or the molecular layers, also contain rIRK3 mRNA. The pattern of rIRK3 mRNA distribution is similar but not identical to that of KGA mRNA that encodes a G protein-coupled inwardly rectifying K^+ channel. A main difference is observed in the cerebellar cortex where KGA mRNA is found in the granule cell layer but not in Purkinje cells [22].

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